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# Epigenetic silencing of genes enhanced by collective role of reactive oxygen species and MAPK signaling downstream ERK/Snail axis: Ectopic application of hydrogen peroxide repress CDH1 gene by enhanced DNA methyltransferase activity in human breast cancer

Nibedita Pradhan, Sabnam Parbin[1], Swayamsiddha Kar[2], Laxmidhar Das, Kirtana R., Suma Seshadri G., Dipta Sengupta[3], Moonmoon Deb[4], Chahat Kausar, Samir Kumar Patra*

*Epigenetics and Cancer Research Laboratory, Biochemistry and Molecular Biology Group, Department of Life Science, National Institute of Technology, Rourkela, Odisha 769008, India*

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ABSTRACT

Loss of E-cadherin and epithelial to mesenchymal transition (EMT) are key steps in cancer progression. Reactive oxygen species (ROS) play significant roles in cellular physiology and homeostasis. Roles of E-cadherin (CDH1), EMT and ROS are intriguingly illustrated in many cancers without focusing their collective concert during cancer progression. We report that hydrogen peroxide ($H_2O_2$) treatment modulate CDH1 gene expression by epigenetic modification(s). Sublethal dosage of $H_2O_2$ treatment decrease E-cadherin, increase DNMT1, HDAC1, Snail, Slug and enrich H3K9me3 and H3K27me3 in the CDH1 promoter. The effect of $H_2O_2$ was attenuated by ROS scavengers; NAC, lupeol and beta-sitosterol. DNMT inhibitor, AZA prevented the $H_2O_2$ induced promoter-CpG-island methylation of CDH1. Treatment of cells with U0126 (inhibitor of ERK) reduced the expression of DNMT1, Snail and Slug, increased CDH1. This implicates that CDH1 is synergistically repressed by histone methylation, DNA methylation and histone deacetylation mediated chromatin remodelling and activation of Snail and Slug through ERK pathway. Increased ROS leads to activation of epigenetic machineries and EMT activators Snail/Slug which in their course of action inactivates CDH1 gene and lack of E-cadherin protein promotes EMT in breast cancer cells. ROS and ERK signaling facilitate epigenetic silencing and support the fact that subtle increase of ROS above basal level act as key cell signaling molecules. Free radical scavengers, lupeol and beta-sitosterol may be tested for therapeutic intervention of breast cancer. This work broadens the amplitude of epigenome and open avenues for investigations on conjoint effects of canonical and intrinsic metabolite signaling and epigenetic modulations in cancer.

## 1. Introduction

Cancer is a heterogeneous group of disease associated with genetic alternations, oncogene activation and tumor suppressor genes silencing [1]. Recent evidences suggest that, abnormalities or aberrant regulation of epigenetic modifications contribute significantly in tumorigenesis [2–4]. Epigenetic modifications involving DNA methylation, histone modification and micro RNA mediated gene regulation affects cellular

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*Abbreviations:* DNMTs, DNA methyltransferases; HDACs, histone deacetylaes; ROS, reactive oxygen species; AZA, 5-AZA-Deoxycytidine; TSA, Trichostatin A; NAC, N-Acetyl-cysteine; DMEM, Dulbecco's-modified Eagle's medium; L-15, Leibovitz's medium; FBS, Fetal bovine serum; DCFDA, 2′,7′ dichlorofluorescin diacetate; CAV1, Caveolin1; nCLU, Nuclear Clusterin

* Corresponding author.

*E-mail address:* samirp@nitrkl.ac.in (S.K. Patra).

[1] Current addresses: Department of Biochemistry, Indian Institute of Science, Bangalore, Bengaluru-560012, India.

[2] Current addresses: Department of Biological Sciences, Indian Institute of Science Education and Research Bhopal, Bhopal Bypass Road, Bhauri, Bhopal 462 066, Madhya Pradesh, India.

[3] Current addresses: MRC Human Genetics Unit (Professor Wendy Bickmore's Group), MRC Institute of Genetics & Molecular Medicine, The University of Edinburgh, Western General Hospital, Crewe Road South, Edinburgh EH4 2XU, Scotland, UK.

[4] Current addresses: Biomedical Science (Dr. Hiranmoy Das's Laboratory), Texas Tech University Health Science Center, Amarillo, Texas, USA.

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**Table 1**
Genes tested,and their Primer sequences and size of the products.

| Gene | Primer sequence | Amplicon size (bp) | Annealing temperature |
|---|---|---|---|
| DNMT1 | F 5′-GGCTGAGATGAGGCAAAAAG-3′<br>R 5′-ACCAACTCGGTACAGGATGC-3′ | 112 | 55 °C |
| DNMT3A | F 5′-AATGTGAATCCAGCCAGGAAAGGC-3′<br>R 5′-ACTGGATTACACTCCAGGAACCGT-3′ | 191 | 60 °C |
| DNMT3B | F5′TATTGATGAGCGCACAAGAGAGC-3′ R 5′-GGGTGTTCCAGGGTAACATTGAG-3′ | 111 | 60 °C |
| E-cadherin | F 5′-CGAGAGCTACACGTTCACGG-3′<br>R 5′-GGGTGTCGAGGGAAAAATAGG-3′ | 119 | 60 °C |
| Snail | F 5′-TCTAGGCCCTGGCTGCTACAA-3′<br>R 5′-ACATCTGAGTGGGTCTGGAGGTG-3′ | 131 | 60 °C |
| OCT4 | F 5′-AGCAAAACCCGGAGGAGT-3′<br>R 5′-CCACATCGGCCTGTGTATATC-3′ | 114 | 60 °C |
| SOX2 | F 5′-GGAAATGGAGGGGTGCAAAAGAGG-3′<br>R 5′-TTGCGTGAGTGTGGATGGGATTGGTG-3′ | 150 | 58.0 °C |
| CAV1 | F 5′-ACCCACTCTTTGAAGCTGTTG-3′<br>R 5′-GAACTTGAAATTGGCACCAGG-3′ | 139 | 60 °C |
| nCLU | F 5′-AAGAAAGAGGATGCCCTAAATGAG-3′<br>R 5′-TTCATGCAGGTCTGTTTCAGG-3′ | 130 | 60 °C |
| GAPDH | F 5′-GGAGCGAGATCTCCCCAAAAT-3′<br>R 5′-GGCTGTTGTCATACTTCTCATGG-3′ | 197 | 60 °C |

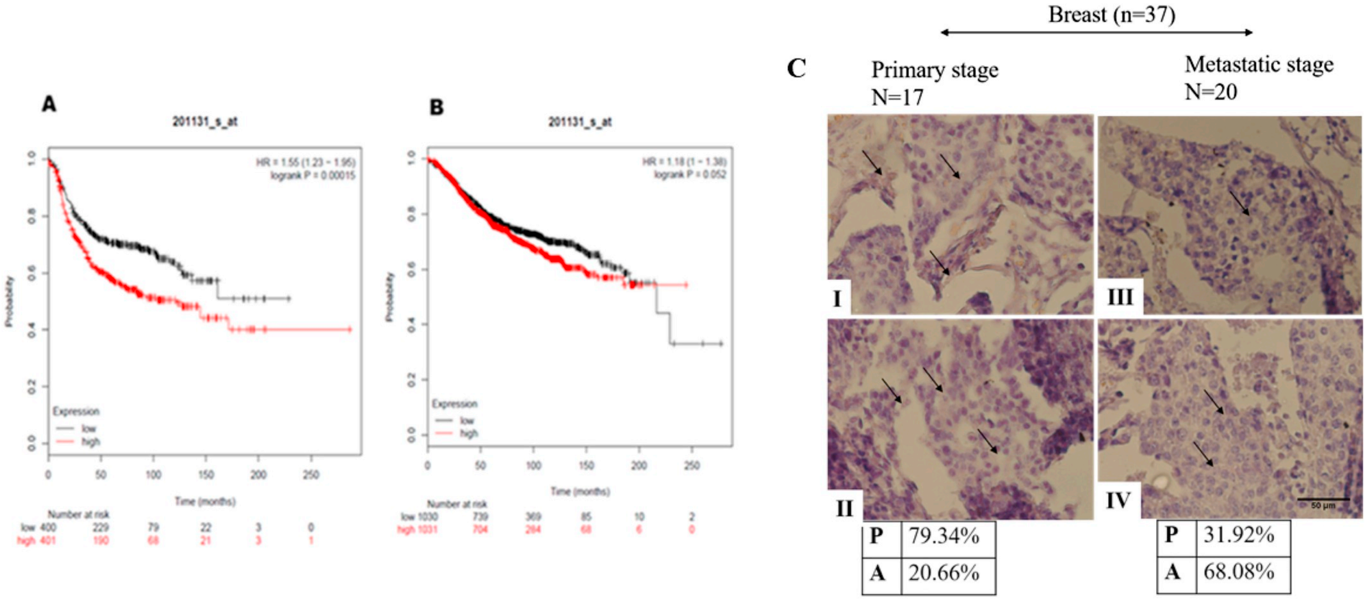

**Fig. 1.** Overall survival analysis (Kaplan-Meier) of patients: using estrogen negative tissue samples (*n* = 801; P = .00015 and HR = 1.55) showing lower expression of E-cadherin indicating poor survival of patients (A), and using estrogen positive tissue samples (*n* = 2061; P = .052 and HR = 1.18) showing lower expression of E-cadherin indicating poor survival of patients (B). It is clear from the plots that survival rate is poor when there is lower expression of CDH1 gene whether estrogen positive or negative.
Analysis of E-cadherin expression in breast cancer tissue samples: Immunohistochemical analysis of the E-cadherin expression in primary (I and II) and metastasis (III and IV) tissue sample. Both show E-cadherin expression (arrow) (C). Metastasis samples show lower level of E-cadherin expression than primary stage tissue sample. Scale bar = 50 µm.

physiology and function of both normal and cancer cells [5–8] Genome wide hypo- and regional (gene promoter specific) hyper-methylation of DNA (aberrant DNA methylation) is one of the most important epigenetic alternations observed in mammalian genome that contributes to the transcriptional silencing of tumor suppressor genes [9,10]. Particularly, the hypermethylation of -CpG-island(s) of gene promoter in association with histone deacetylations plays crucial roles in repression of tumor suppressor genes [11,12] and frequently observed in multiple types of cancer [3,13,14]. Reversible chemical modifications of the chromatin proteins like acetylation and methylation of histones are two important molecular signatures for dynamic regulation of gene function and in this scenario DNA methylation augurs histone deacetylation to precipitate inactive genes. Dysregulation of these modifications are involved with tumor development and cancer progression [15]. Along with DNA methylation and histone modifications, dysregulation of genes that encode the proteins for DNA and histone modifications [for examples, DNA methyltransferases (DNMTs) and Histone deacetylases (HDACs)] also play active roles in tumor development and cancer progression affecting cell survival, migration and invasion [16,17]. E-cadherin, a cell surface glycoprotein and member of cadherin family, plays important role in tissue integrity and reduces cell motility; hence, it is termed as tumor suppressor gene. E-cadherin also plays important roles in cellular migration, maintaining cell polarity and structure of normal epithelial cells [18]. Downregulation of E-cadherin is associated with enhanced epithelial to mesenchymal transition (EMT), aggressiveness, invasion and metastasis of breast cancer cells and well documented that CDH1 gene is inhibited by activation, nuclear translocation and binding of transcription factors such as Snail, Slug and Twist on the

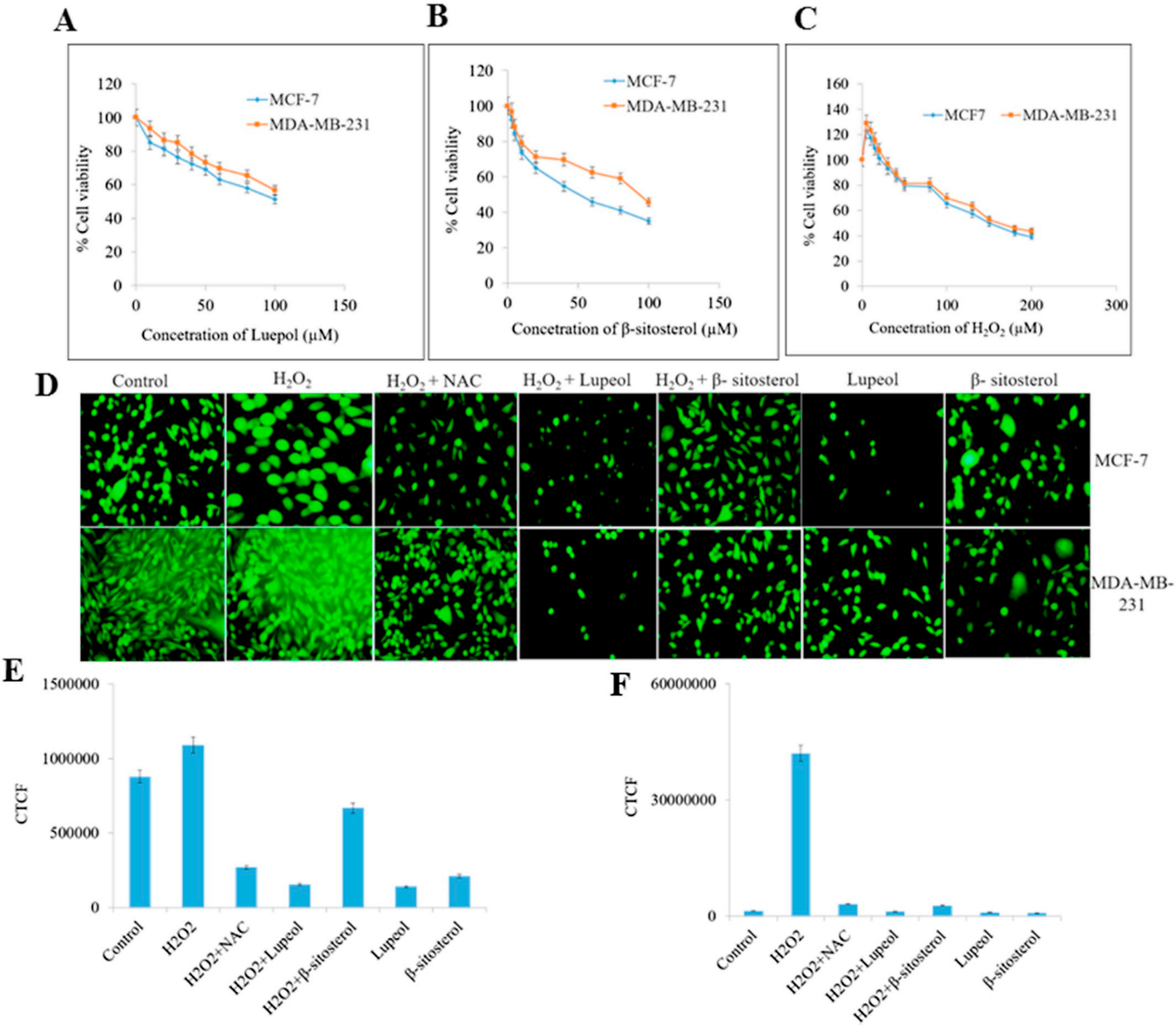

**Fig. 2.** Effect of free radical scavengers, Lupeol (A) and beta-sitosterol (B) on viability and growth of MCF-7 and MDA-MB-231 breast cancer cells. Both cell lines were treated with specific dosages of the respective drugs as marked in the figure. Cytotoxicity of drugs was determined by MTT assay. Effect of free radical inducer $H_2O_2$ on proliferation of MCF-7 and MDA-MB-231 breast cancer cells(C). $H_2O_2$ at lower concentration (1–15 µM) promotes cell proliferation, whereas higher concentration (20–180 µM) inhibits cell proliferation. Data are expressed as mean ± SEM., $n = 3$, $p < .05$.

Detection of $H_2O_2$ induced ROS production by fluorescence measurement using DCFDA dye (D). Important to note the reduction of free radical generation by same amount of $H_2O_2$ when antioxidants NAC, lupeol and beta sitosterol were simultaneously used in both MCF-7 and MDA-MB-231 breast cancer cells. MCF-7 and MDA-MB-231 cells were treated with $H_2O_2$ in the presence or absence of different drugs and NAC for 24 h and ROS level was detected using DCFDA dye by fluorescence microscopy as seen in the representative photographic images. Data are expressed as mean ± SEM., $n = 3$, $p < .05$.

Fluorescence intensity reflects the amount of ROS production in terms of CTCF (Corrected Total Cell Fluorescence) in MCF-7 (E) and MDA-MB-231(F) cell lines. Data are expressed as mean ± SEM., $n = 3$, $p < .05$.

promoter of CDH1 [19–23]. We and other laboratories reported that, CDH1 gene silencing by promoter hypermethylation is the main cause of E-cadherin protein scarcity in breast cancer [24–26].

Reactive oxygen species (ROS), like hydrogen peroxide ($H_2O_2$) play a major role in carcinogenesis by DNA damage and activation of pro-inflammatory signaling pathways. Overproduction and accumulation of ROS is associated with increase in nucleotide modifications, DNA strand break, deletions and chromosomal rearrangement. Depending on its intracellular concentration and localization, $H_2O_2$ exhibits either pro- or anti-apoptotic activities. In comparison to normal cells, cancer cells are characterized by an increased $H_2O_2$ production rate and an impaired redox balance thereby affecting the microenvironment as well as the anti-tumoral immune response. Emerging evidences suggests ROS acts as critical signaling stimuli involving various cellular functions such as apoptosis, migration, invasion and EMT in the tumor micro-environment [27,28]. Small molecule second messengers (for examples, calcium and hydrogen peroxide) propagate information quickly owing to their much larger diffusion coefficient [29,30]. The roles of E-cadherin, EMT and ROS in many cancers are illustrated discreetly. The molecular mechanisms, signaling and/or network interdependence of their action in tumor development and cancer progression are not studied adequately. To decipher the mechanisms and pathways of actions further, we thoroughly investigate whether ROS, like hydrogen peroxide may modulate E-cadherin expression! And if it does so, which

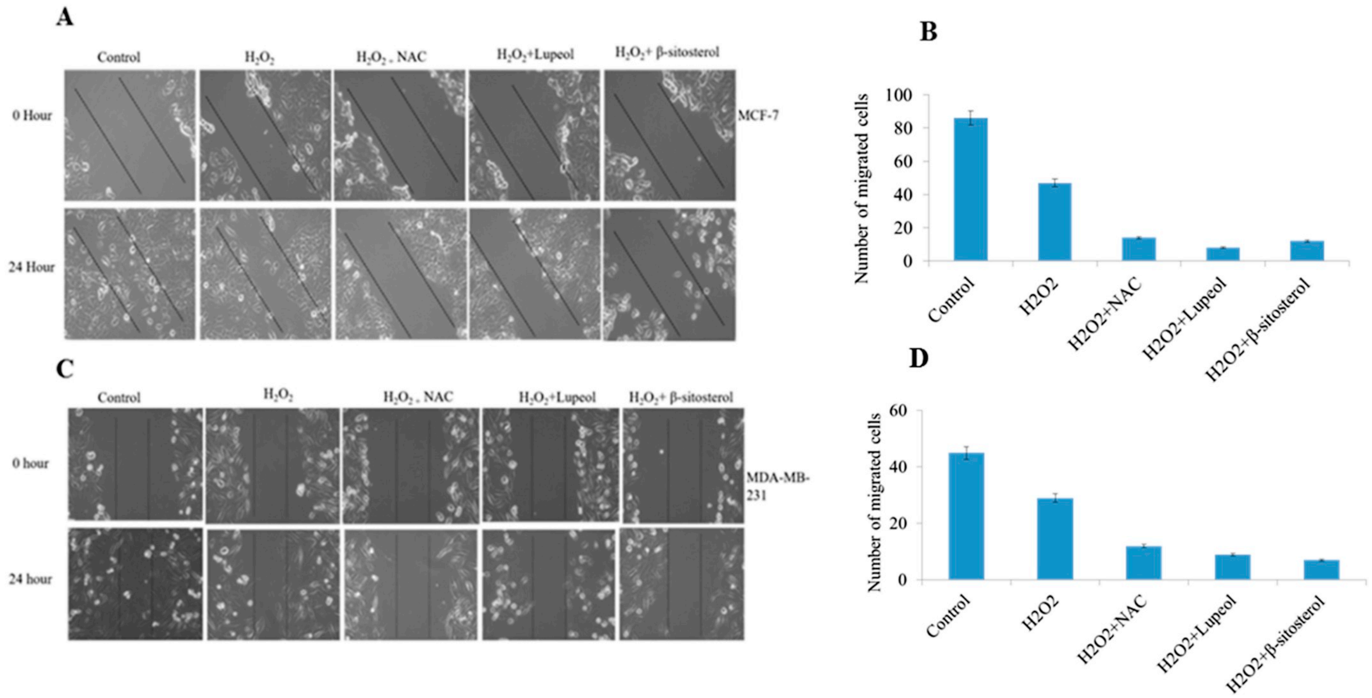

**Fig. 3.** Effect of $H_2O_2$ treatment on cell migration. MCF-7 and MDA-MB-231 cells were exposed to $H_2O_2$ (40 μM as mentioned in the respective images) and it induces cell migration, however, simultaneous treatment with Lupeol and beta-sitosterol at their respective $IC_{30}$ prevents cell migration (instead of $IC_{50}$ we used the concentrations of drugs at their respective $IC_{30}$, see the text for further details). The monolayer cultures were scratched and the wound areas were photographed at the indicated time points. Scratch Assay showing the cellular migration in control and drug treated MCF-7(A) and MDA-MB-231(C) cell lines. Graphical representation of number of migrated cells in control and drug treated MCF-7(B) and MDA-MB-231(D) cell lines. Data are expressed as mean ± SEM., $n = 3, p < .05$.

mechanism(s)/epigenetic modification(s) are involved in the inactivation of CDH1 gene promoter? We have executed bioinformatics analyzes and cell culture experiments. Cells were cultured and treaded with sub lethal dosage of $H_2O_2$ mixed in culture media. The expression profile of E-cadherin, DNA methyltransferase 1 (DNMT1), histone deacetylase 1 (HDAC1), and EMT factors Snail and Slug were recorded. Of notice, CDH1 was silenced, and DNMT1, HDAC1, Snail and Slug were overexpressed by $H_2O_2$ treatment. Application of ROS scavengers; NAC, lupeol and beta sitosterol attenuated this effect. Additionally, application of DNMT inhibitor 5-Aza-2′-deoxycytidine (AZA) prevented the $H_2O_2$ induced downregulation of E-cadherin, grossly suggesting promoter -CpG- methylation of CDH1 gene. Methylation of CDH1 was confirmed by MS-PCR experiments. Then we used ERK inhibitor, U0126 in cell culture which reduced the expression of DNMT1, Snail and Slug, but expression of CDH1 was enhanced. This simple experiment depicts that the expression of CDH1 is synergistically regulated by ERK pathway downstream effectors DNMT1 and HDAC1 overexpression (causing CDH1 promoter DNA methylation and inactivation), Snail and Slug over expression and occupancy of CDH1 promoter for keeping it silent.

Hence, we conclude that, enhancement of hydrogen peroxide level in cell by deregulation of intrinsic metabolism may induce: (i) overexpression of epigenetic modifiers, including DNMT1 and HDAC1; (ii) inhibit gene expression, including tumor suppressor genes (for example, CDH1 gene) and thus reduce the amount of proteins those maintain cellular homeostasis and cell-cell contact (for example, E-cadherin protein); and (iii) enhance expression of EMT inducer genes, including Snail and Slug in human breast cancer.

## 2. Materials and methods

### 2.1. Reagents and antibodies

All the chemicals used were molecular biology grade. Culture media, FBS and trypsin were purchased from Himedia and antibiotic from GIBCO. All the routine chemicals were purchased from Sigma. Stock solutions of 5-AZA-2′-Deoxycytidine (Sigma Aldrich), TSA (Sigma Aldrich) and U016 (Santa Cruz, Biotechnology) was prepared by dissolving in DMSO and stored at −20 °C and diluted in fresh medium immediately before use. Similarly, stock solutions of Lupeol and beta sitosterol were prepared by dissolving in 100% ethanol and dimethyl sulfoxide (DMSO) in 1:1 ratio and used immediately or stored at −20 °C for further use. For western blot analysis Rabbit polyclonal anti-DNMT1, Rabbit polyclonal anti-HDAC1 and mouse monoclonal anti-beta-actin antibodies were purchased from Santa-Cruz Biotechnology.

DNMT1 siRNA and control siRNA were also purchased from Santa-Cruz Biotechnology. Rabbit polyclonal anti-HDAC2 and Rabbit polyclonal anti-E cadherin, were purchased from Abcam. Primer pairs for qRT-PCR were synthesized from Sigma. For chromatin immunoprecipitation assay Rabbit polyclonal anti-H3K4me1 (Abcam), Rabbit polyclonal anti-H3K9me3 (Abcam) and Rabbit polyclonal anti-H3K27me3 (Abcam) were used.

### 2.2. In-silico analysis of E-cadherin expression from publicly available databases

Pre-experimental in-silico analysis of the expression profile of E-cadherin in breast carcinoma was evaluated employing publicly available online bioinformatics tools like Kaplan-Meier plotter (KM plot). Overall survival curve of patients having altered E-cadherin expression was computed from Kaplan-Meier graphs by using online KMPLOT tool for individual estrogen positive and estrogen negative patients.

### 2.3. Tissue samples and immunohistochemistry

Tissue samples of breast cancer patient were collected from Dr. Trivedi and Roy Diagnostic Laboratory (Kolkata India), processed to prepare parafilm coated cubes and sliced into thin pieces of 0.5 μm

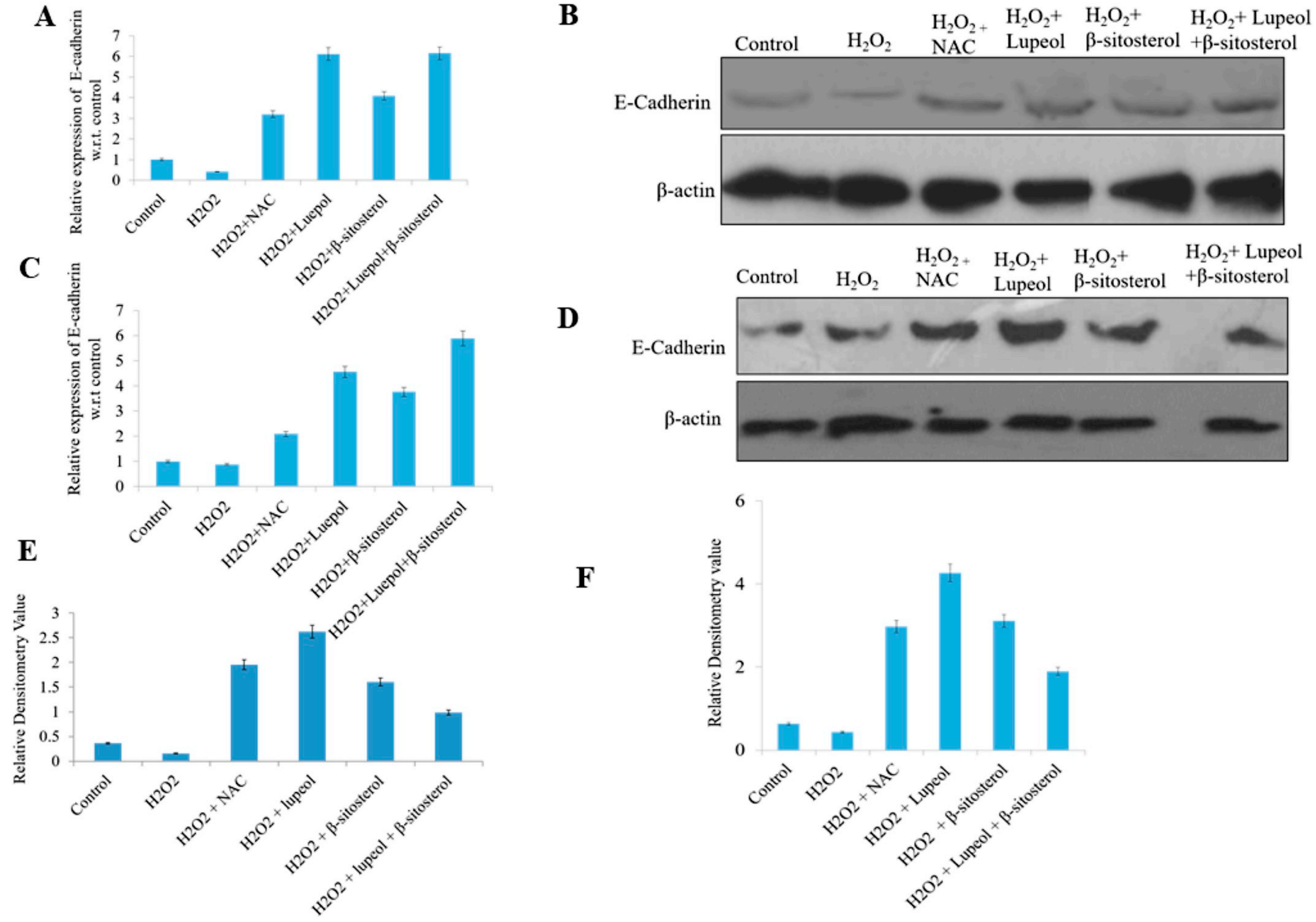

**Fig. 4.** CDH1 expression is downregulated by $H_2O_2$ treatment. E-Cadherin expression is analyzed by qRT-PCR (A) and western blot (B) in $H_2O_2$, lupeol and beta-sitosterol treated MCF-7 cell line; and qRT-PCR (C) and western blot (D) in $H_2O_2$, lupeol and beta sitosterol treated MDA-MB-231cell line. It is clear from the figures that CDH1 gene is downregulated in $H_2O_2$ treated cells; however, treatment with the antioxidants NAC, lupeol and beta-sitosterol significantly upregulated CDH1. Results of Densitometric scanning for quantitating E-cadherin protein after normalization with beta-actin in MCF-7(E) and MDA-MB-231 (F) cell lines. beta-actin was used as internal control. Data are expressed as mean ± SEM., $n = 3$, $p < .05$.

thickness. Fine pieces were mounted and fixed properly. It was subjected to antigen retrieval buffer followed by blocking with endogenous peroxidase activity and rinsed with Tris-buffered saline (TBS) containing 0.25% TritonX-100 (TBST). Tissue samples were incubated with Rabbit polyclonal anti-Ecadherin (Abcam) overnight at 4 °C and then washed properly with TBST before incubated with secondary antibody for 1 h at room temperature. Finally, slides were rinsed with TBST and treated with 3,3′-diaminobenzidine tetrahydrochloride (Sigma) for colour development. Hematoxylin was used for nuclear staining.

### 2.4. Cell lines and cell culture

The human breast cancer cell line MCF-7 and MDA-MB-231 were purchased from National Centre for Cell Science (NCCS), Pune, India and were cultured in Dulbecco's-modified Eagle's medium (DMEM) and Leibovitz's medium (L-15) respectively, supplemented with 10% (v/v) Fetal bovine serum (FBS), penicillin (100 unit/ml) and streptomycin 0.1 mg/ml. Cells were cultured in a humidified atmosphere of 5% $CO_2$ at 37 °C. The cells were harvested by trypsinization and number of living cells was calculated by trypan blue staining (0.2% v/v) using a haemocytometer.

### 2.5. Drug treatment and cell viability assay

To determine the effect of drugs on cellular viability and to find out the sublethal concertation of drugs MTT assay was done. About 5000 live cells/well were seeded in a 96-well plate for viability assay. Trypan blue was used to determine cell viability. After 24 h of incubation of MCF-7 and MDA-MB-231 cells, different concentrations of drugs were treated to determine the minimum inhibitory concentration ($IC_{30}$ value). After 24 h of drug treatment MTT solution (0.8 mg/ml dissolved in serum free medium) was subjected and further incubated for 3 h in dark at 37 °C. The media containing MTT was removed and DMSO was added followed by incubation for 15 min at dark. Then the absorbance was measured at 595 nm and mean of five replicas was taken to obtain $IC_{30}$ value for subsequent experiments. For DNA methylation inhibition by AZA (15 μM) treatment, cells were cultured for 72 h.

### 2.6. Cell migration by scratch/wound healing assay

For analysis of cell migration, MCF-7 and MDA-MB-231 cells were seeded in 6-well plate and maintained upto 90% confluency. A sterile 200 μl pipette tip was used to make wound by straight scratch. Cells were then washed with PBS to remove the cell debris and suitable drug was treated by adding fresh complete media. After 24 h photograph was taken to visualize cell migration and width of the wound was measured.

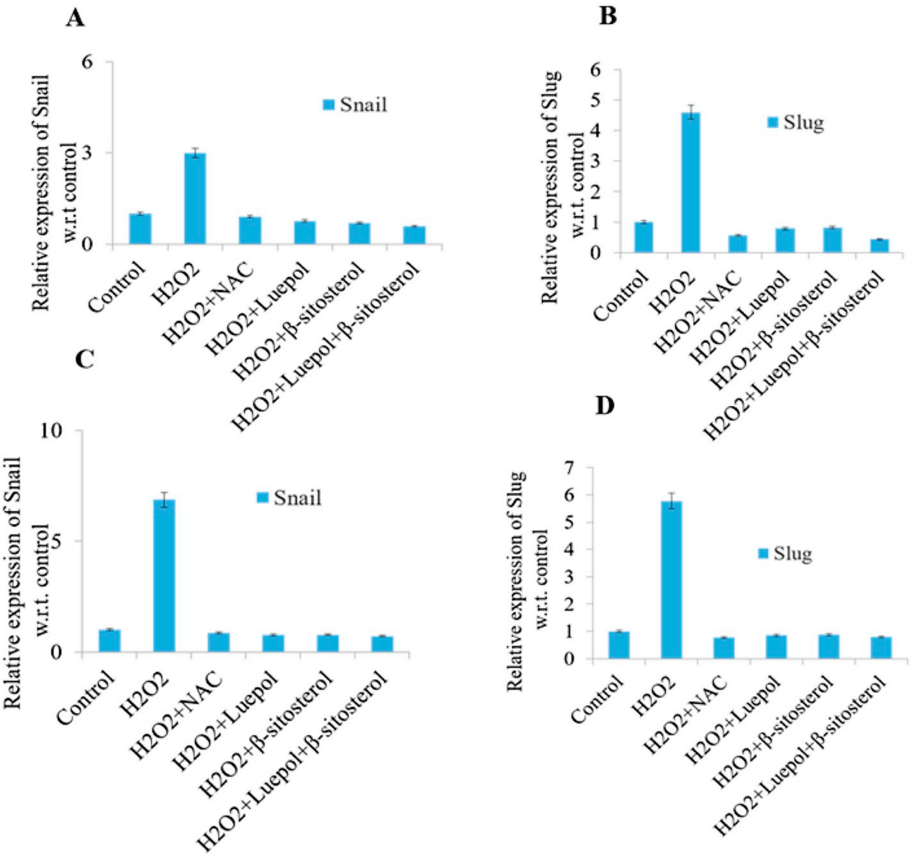

**Fig. 5.** Effect of $H_2O_2$ induced ROS on the expression of Snail and Slug. qRT-PCR analyses of Snail (A) and Slug (B) expression in MCF-7 cell line; Snail (C) and Slug (D) expression in MDA-MB-231cell line after treatment with $H_2O_2$, lupeol and beta sitosterol. $H_2O_2$ treatment shows higher expression of Snail and Slug as compared to control (untreated). Data are expressed as mean ± SEM., n = 3, $p < .05$.

### 2.7. RNA extraction and quantitative expression analysis of mRNA by real time PCR

MCF-7 and MDA-MB-231 cells treated with different drugs were harvested and properly rinsed in PBS for RNA isolation. RNA was extracted using Trireagent according to the manufacturer's instructions. c-DNA was synthesized taking total RNA(1 μg) with oligodT using RevertAid First strand c-DNA synthesis kit (Thermo Scientific) as per the manufacturer's instruction. To study expression of genes qRT-PCR was done using specific primer pairs (detailed mentioned in Table 1) and SYBR Green JumpStart™Taq Ready mix in Realplex4 eppendrof system. PCR was started with 5 min at 95 °C for denaturation, followed by 40 cycles taking appropriate primer sets (Table 1). A final extension step at 72 °C for 7 min was included after the final cycle to complete polymerization. Number of cycles was optimized within the exponential phase of amplification. The expression of each gene was normalized with GAPDH as internal control.

### 2.8. Western blot analyses

Cells treated with different drugs were harvested and total cellular proteins were extracted using RIPA lysis buffer containing protease inhibitor cocktail (Sigma) and centrifuged for 15 min at 14000*g* at 4 °C. The concentration of protein was quantified. Then equal amount of proteins from each sample were loaded in 10% SDS-polyacrylamide gel and electroblotted to PVDF membrane (Millipore) followed by proper electrophoresis. The membrane was blocked with 3% BSA in PBST for 2 h at RT. The blots were then incubated with primary antibody at 4 °C for overnight and incubated in HRP-conjugated secondary antibody after proper washing. Then Immunoreactive proteins were developed and detected in X-ray film using ECL (Thermo Scientific). Band density values were normalized with beta-actin using the Gel documentation system and analyzed by image J software.

### 2.9. siRNA transfection and knockdown of DNMT1

To observe the role of DNMT1, Knockdown of DNMT1 was done using DNMT1 siRNA. To determine the effective concentration of siRNA that completely abolished gene and protein expression transfection was done using specific siRNA against DNMT1. Apparently, 300 nM of DNMT1 siRNA efficiently inhibits the expression of DNMT1 at the protein level. Thus transfection was done using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. After 6 h of transfection, media was changed by complete media and cells were further incubated for 24 h before processing.

### 2.10. Methylation specific PCR

Genomic DNA was isolated from drug treated cells and MS-PCR was done taking methylation specific primer and using EpiTect Bisulphite Kit (Qiagen), according to manufacturer's instruction. Briefly, 2 μg of genomic DNA was bisulfite converted as per the given protocol. Bisulphite converted DNA was further used as template for PCR reaction. For amplification, primer pairs used for methylated CDH1 were forward: 5′-TAACTACAACCAAATAAACCCCG-3′ and reverse: 5′-TCGA ATTTAGTGGAATTAGAATCGT-3′. Similarly, primer pairs used for unmethylated E-cadherin were forward: 5′-TAACTACAACCAAATAAACC CCAAA-3′ and reverse: 5′-TTGAATTTAGTGGAATTAGAATTGT -3′ used to amplify the region located at the 5′ end of E-cadherin promoter region having annealing temperature of 50 °C.

### 2.11. Fluorescence detection of intracellular ROS production

MCF-7 or MDA-MB-231 cells were seeded in 6-well plate. At proper confluence cells were treated with 40 μM $H_2O_2$ and/or different drugs for 24 h. Cells were washed in PBS and fixed with ice cold methanol for 10 min. Then cells were incubated with reagent 2′,7′–dichlorofluorescin

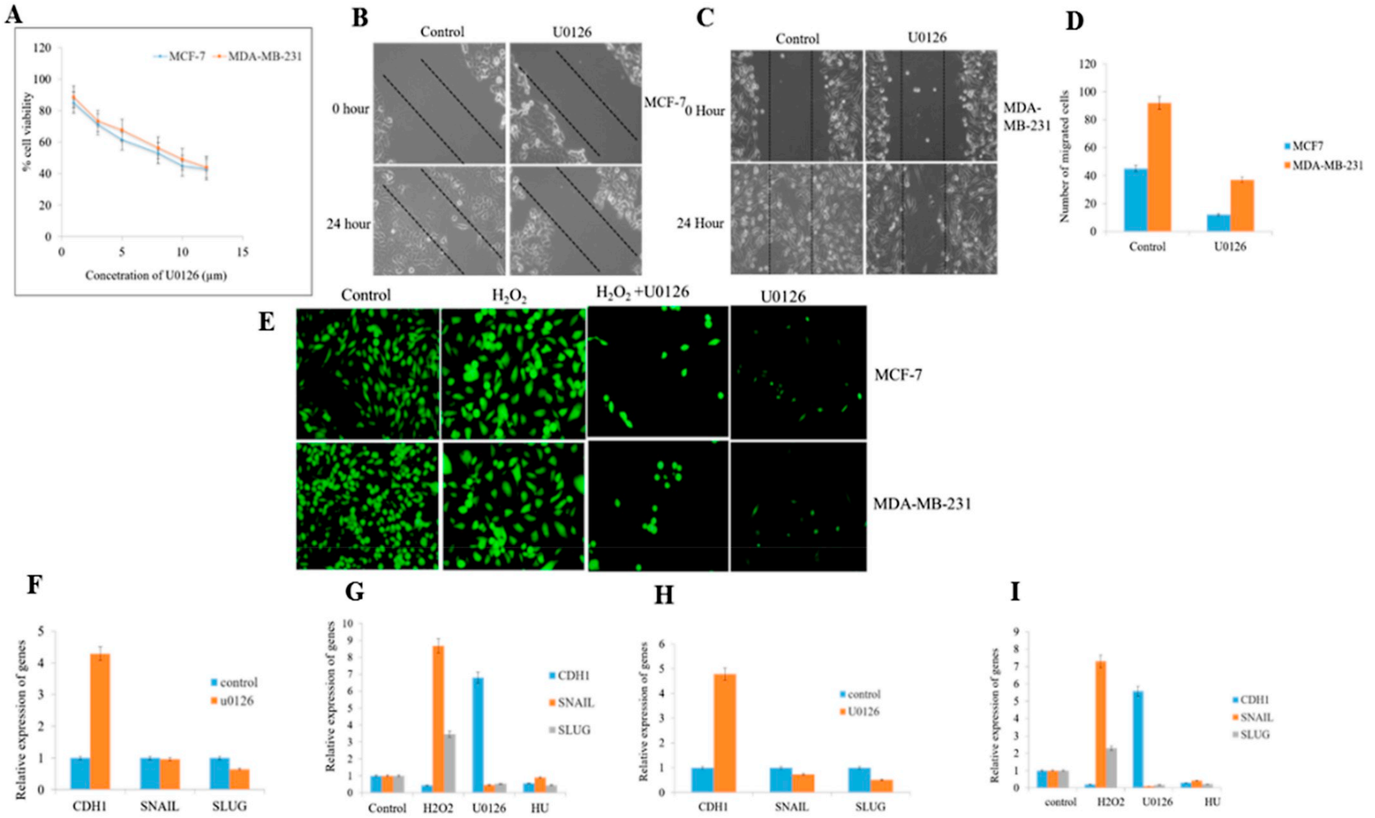

**Fig. 6.** Role of ERK signaling pathway on CDH1 expression. MTT assay for determining the $IC_{30}$ value of ERK inhibitor, U0126 in both MCF-7 and MDA-MB-231 breast cancer cells (A). U0126 inhibits cellular migration in MCF-7(B) and in MDA-MB-231(C) breast cancer cell lines. Briefly, both MCF7 and MDA-MB-231 cells were exposed to U0126 treatment at their respective $IC_{30}$ value. The monolayer cultures were scratched and the wound areas were photographed at the indicated time points. Graphical representation of the number of migrated cells in control and drug treated MCF-7 and MDA-MB-231 cell lines (D). Detection of $H_2O_2$, U0126 and $H_2O_2$ + U0126 induced ROS production by fluorescence measurement using DCFDA dye. $H_2O_2$ treatment increased the amount of ROS production; however simultaneous treatment with $H_2O_2$ + U0126 has no such effect on ROS production (E)
Analyses of E-Cadherin, Snail and Slug expression by qRT-PCR after treatment with U0126 (F) and $H_2O_2$ + U0126 (G) in MCF-7 cell line; U0126 (H), and $H_2O_2$ + U0126 (I) in MDA-MB-231 cell lines. It is clear that U0126 upregulates the expression of E-cadherin and downregulates the expression of Snail and Slug in MCF-7 and MDA-MB-231 cells. $H_2O_2$ treatment decrease CDH1 expression by upregulation of Snail and Slug genes. Interestingly, simultaneous treatment of $H_2O_2$ + U0126 induces the expression of CDH1 and reduced the expression of Snail and Slug as compared to sole $H_2O_2$ treated cells. Data are expressed as mean ± SEM., $n = 3$, $p < .05$.

diacetate (DCFDA) dye for 30 min at 37 °C and intracellular ROS levels were detected. Fluorescence images were taken under epifluorescence microscope (Olympus IX71).

### 2.12. Chromatin immunoprecipitation (ChIP) assay

ChIP was performed with antibody specific for H3K4me1, H3K9me3 and H3K27me3 using an imprint chromatin immunoprecipitation kit (Sigma) according to the manufacturer's instruction. 2 μl of sonicated DNA were used for PCR analysis with PCR conditions 95 °C for 2 min followed by 40 cycles of 95 °C for 20 min with an annealing temperature 54 °C of for 30 s and at a final extension of 72°°C for 30 s. E-cadherin having the primer sequence for sense strand (5′-GGCCGGCAGG TGAAC-3′) and antisense strand (5′-GGGCTGGAGTCTGAACTGAC-3′) producing a fragment of 146 base pair was amplified for analysis. Anti-mouse IgG precipitated DNA was used as template for negative control. Input DNA 1% was used for normalization.

### 2.13. Statistical analysis

All datas are presented as mean ± SEM. Statistical analysis of the data were performed by student's *t*-test using SPSS software. Variations with $p < .05$ were considered as significant value.

## 3. Results

### 3.1. CDH1 expression is reduced in breast cancer and overall survival analysis shows a significant impact

In order to understand the impact of E-cadherin expression on overall survival of patients, free survival analysis from online breast cancer KM plotter was retrieved for both estrogen positive and estrogen negative patients [31]. To determine whether E-cadherin expression is significantly associated with breast cancer prognosis, data-mining of patients' survival outcome with follow-up information were performed. In both cases lower expression of E-cadherin in estrogen negative ($P = .00015$, $HR = 1.55$) (Fig. 1A) and estrogen positive ($P = .052$, $HR = 1.18$) (Fig. 1B) implicated poor survival of patients. This suggests that survival rate is poor when there is lower expression of CDH1 gene whether estrogen positive or negative. To validate the in silico data analysis, we examined the E-cadherin protein expression by immunohistochemistry analysis from a cohort of 37 breast tissues comprising both benign and malignant samples. Immunohistochemistry analysis of tissue samples showed lower expression of E-cadherin in malignant breast tissues, suggesting that lower expression of E-cadherin is associated with progression of breast cancer phenotype (Fig. 1C). To decipher a clear mechanism of CDH1 gene repression we then performed series of experiments and molecular analyzes as follows:

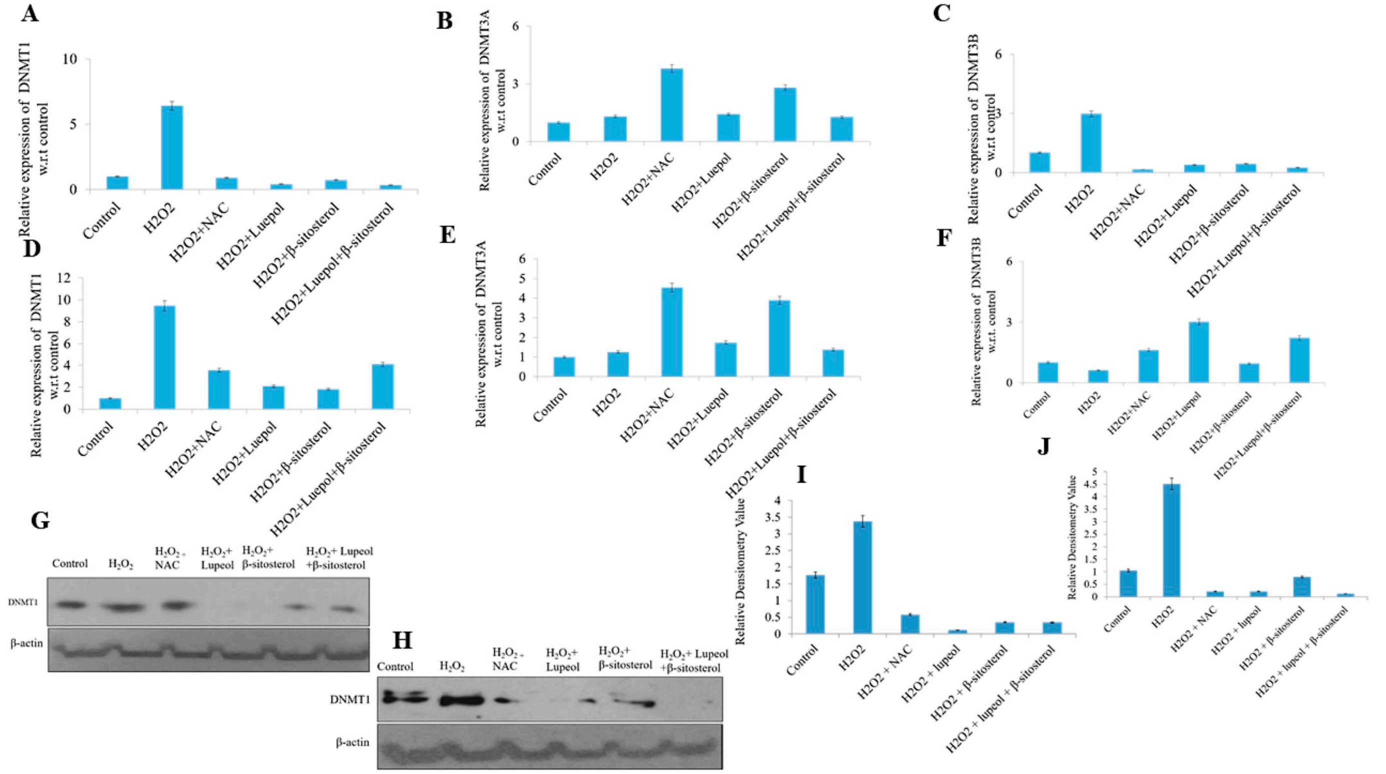

**Fig. 7.** Effect of $H_2O_2$ on the expression DNMTs. qRT-PCR analyses of DNMT1 (A), DNMT3A (B) and DNMT3B (C) in MCF-7 cell line; DNMT1 (D), DNMT3A (E) and DNMT3B (F) MDA-MB-231 cell line respectively after $H_2O_2$, lupeol and beta sitosterol treatments. $H_2O_2$ induced the expression of DNMT1 in both MCF-7 and MDA-MB-231 cells whereas treatments with NAC, lupeol and beta sitosterol inhibit the $H_2O_2$ induced DNMT1 expression as evident at the mRNA level. However, it does not have significant effect on DNMT3A and DNMT3B as compared to control (untreated cells). Data are expressed as mean ± SEM., $n = 3$, $p < .05$.
Western blot analysis of DNMT1 protein after $H_2O_2$, lupeol and beta sitosterol treated MCF-7 cell line (G) and MDA-MB-231 cell line (H). Densitometric scanning of E-cadherin after normalization with beta-actin in MCF-7 (I) and MDA-MB-231 (J) cell lines. beta-actin was used as internal control. Data are expressed as mean ± SEM., n = 3, $p < .05$.

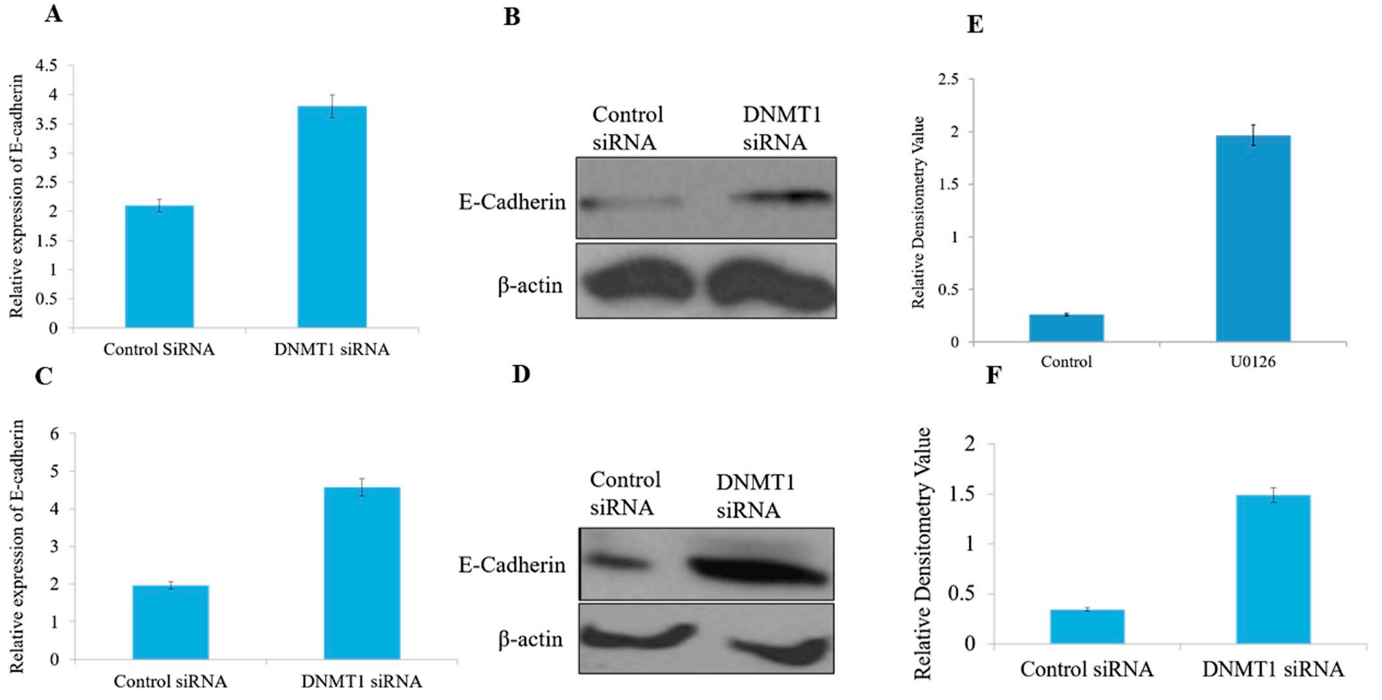

**Fig. 8.** Effect of DNMT1 knockdown on CDH1 expression. CDH1 expression at mRNA level was analyzed by qRT-PCR and protein level by Western blot on treatment with siDNMT1 and control siRNA. siDNMT1 treatment induced the expression of CDH1 gene (A) and protein (B) in MCF-7 cell line; and CDH1 gene (C) and protein (D) in MDA-MB-231cell line as compared to control siRNA treatment. Beta-actin was used as internal control. Densitometric scanning of E-cadherin after normalization with beta-actin in MCF-7 (E) and MDA-MB-231(F) cell lines. Data are expressed as mean ± SEM., n = 3, $p < .05$.

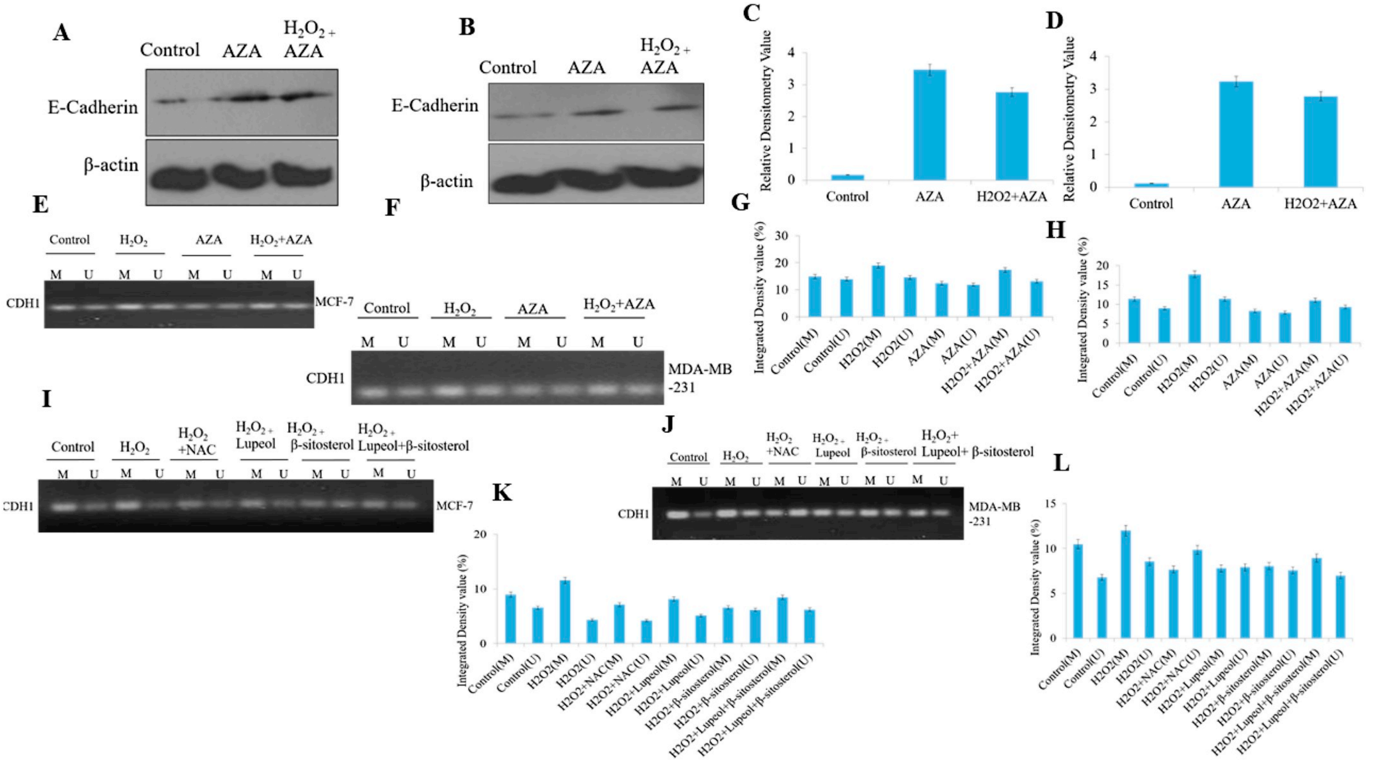

**Fig. 9.** Effect of DNA methylation inhibitor 5-Aza-2′-deoxycytidine (AZA) on CDH1 gene expression. Western blot analysis of E-cadherin in AZA and $H_2O_2$ + AZA treated MCF-7 cell line (A) and MDA-MB-231 cell line (B). Densitometric scanning of E-cadherin after normalization with beta-actin in MCF-7 (C) and MDA-MB-231(D) cell lines. Beta-actin was used as internal control. AZA treatment prevented the $H_2O_2$ induced downregulation of E-cadherin in both MCF-7 and MDA-MB-231 cell line. Methylation specific PCR of E-cadherin promoter in AZA and $H_2O_2$ + AZA treated MCF-7 cells (E) and in MDA-MB-231 cell lines (F). Densitometric scanning of the band showing CDH1 expression in MCF-7 cells (G) and in MDA-MB-231 cell lines (H). Methylation specific PCR of E-cadherin promoter in $H_2O_2$, lupeol and beta sitosterol treated MCF-7 (I) and MDA-MB-231(J) breast cancer cell lines. Densitometric scanning of the band of CDH1 expression in MCF-7 cells (K) and in MDA-MB-231 cell lines (L). Significantly higher methylation of E-cadherin promoter was observed in $H_2O_2$ treated cells compared to untreated one which was inhibited by DNMT inhibitor (AZA) as well as by NAC, lupeol and beta sitosterol. Data are expressed as mean ± SEM., n = 3, $p < .05$.

### 3.2. Effect of drugs on viability of MCF-7 and MDA-MB-231 cancer cells

Effect of drugs, free radical scavengers, on cell viability was analyzed by MTT assay. MCF-7 and MDA-MB-231 breast cancer cells were treated with different concentration of lupeol and beta-sitosterol for 24 h to determine their effect on cell viability. Lupeol and beta-sitosterol inhibit cell viability in a concentration dependent manner (Fig. 2A and B). The $IC_{30}$ value of lupeol and beta-sitosterol was found to be 60 μM and 40 μM respectively for MCF-7. Similarly, for MDA-MB-231 the $IC_{30}$ value of lupeol and beta-sitosterol was 80 μM and 60 μM respectively. Instead of $IC_{50}$ we used $IC_{30}$ to have more viable cells for respective molecular analyses and continued in subsequent experiments unless otherwise mentioned.

### 3.3. Effect of $H_2O_2$ on viability of MCF-7 and MDA-MB-231 breast cancer cells

Effect of $H_2O_2$ on MCF-7 and MDA-MB-231 cells was analyzed using MTT cell viability assay, for which cells were treated with lower range concentration of $H_2O_2$ (1–15 μM) and a higher range concentration of $H_2O_2$ (20–180 μM) for 24 h and MTT Assay was done. Results obtained here shows $H_2O_2$ at higher concentration inhibits cell proliferation, whereas, at lower concentration it promotes cell proliferation (Fig. 2C). Based on this results 40 μM concentration was taken as a moderate range for further experiments which induce cell proliferation.

### 3.4. Lupeol and beta-sitosterol inhibit $H_2O_2$ induced ROS production in breast cancer cell lines

To determine the antioxidant property of Lupeol and beta-sitosterol, MCF-7 and MDA-MB-231 cells were treated simultaneously with a suitable concentration of $H_2O_2$, Lupeol and beta-sitosterol for 24 h. In addition, NAC was used as a reference antioxidant to compare its effect with Lupeol and beta sitosterol. Formagenic compound (Fluorescent compounds) i.e. DCF (2′, 7′-dichlorofluorescein) visualized under Epifluorescent (Olympus IX71) microscope shows treatment with the antioxidant NAC (at 10 μM) concentration as well as lupeol and beta sitosterol produced significantly less ROS compared to $H_2O_2$ treated and control MCF-7 and MDA-MB-231cells (Fig. 2D, E and F).

### 3.5. Lupeol and beta-sitosterol inhibit $H_2O_2$ induced migration of breast cancer cells

MCF-7 and MDA-MB-231cells exhibited $H_2O_2$ induced of migration at 40 μM concentration of $H_2O_2$. Further, to visualize the antagonistic effect of lupeol and beta-sitosterol on $H_2O_2$ induced cellular migration; cells were treated with $H_2O_2$, lupeol and beta-sitosterol as done in previous experiments. Results indicate that $H_2O_2$ induced cellular migration of MCF-7 (Fig. 3A and B) and MDA-MB-231 (Fig. 3C and D) cells were inhibited upon treatment with NAC, well-known antioxidant as well as lupeol and beta-sitosterol.

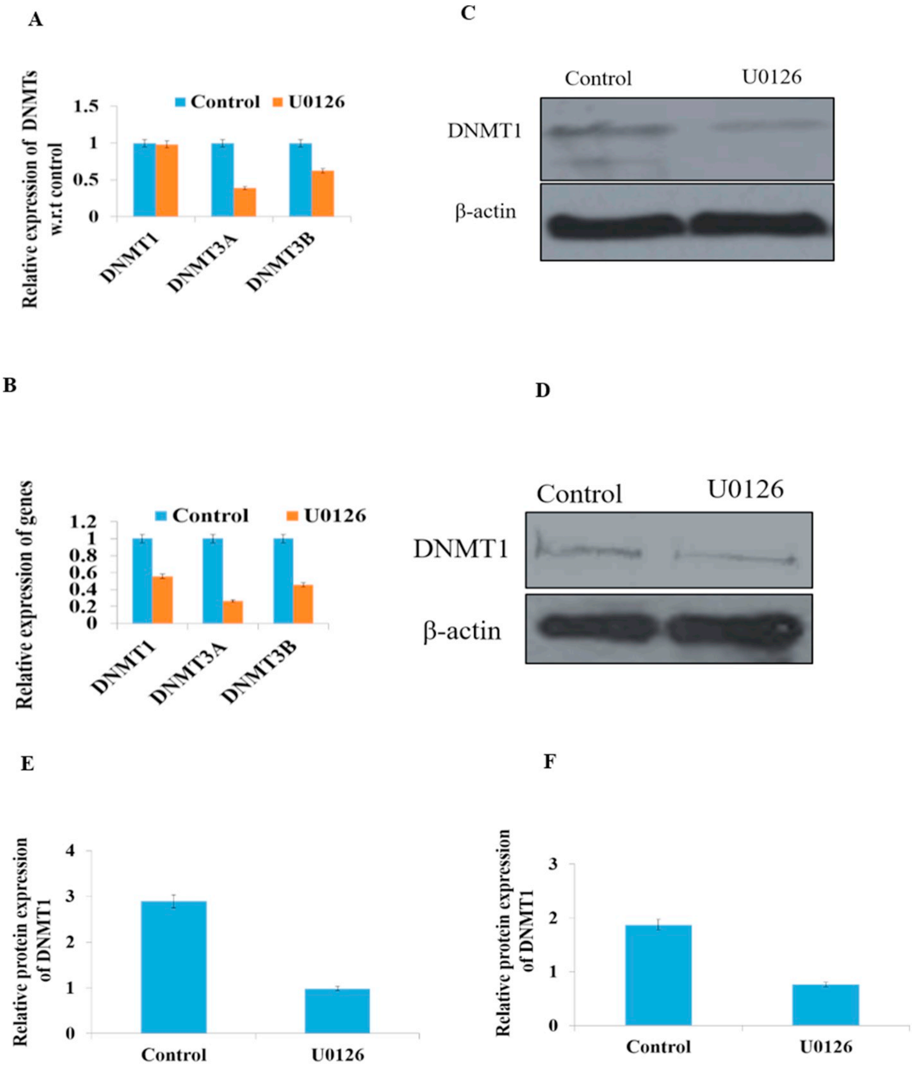

**Fig. 10.** Role of ERK pathway on DNA methylation. DNMTs expression at mRNA level was analyzed by qRT-PCR in U0126 treated MCF-7 cell lines (A) and in MDA-MB-231 cell lines (B). Protein level was analyzed by western blot in U0126 treated MCF-7 cell line (C) and RT-PCR in MDA-MB-231 cell lines (D). Densitometric scanning of DNMT1 after normalization with beta-actin in MCF-7(E) and MDA-MB-231(F) cell lines. Treatment with U0126 reduced the protein expression of DNMT1 in both MCF-7 and MDA-MB-231 breast cancer cells. Data are expressed as mean ± SEM., n = 3, $p < .05$. beta-actin was used as internal control.

### *3.6. Lupeol and beta-sitosterol treatment reduce the inhibitory effect of $H_2O_2$ on CDH1*

To further confirm the role of ROS on cell migration, expression of *E*-cadherin after $H_2O_2$ treatment was analyzed, since E-cadherin plays an important role in cellular migration. $H_2O_2$ at 40 μM concentration significantly downregulated the expression of CDH1 at m-RNA and protein level in both MCF-7 (Fig. 4A, B and E) and MDA-MB-231(Fig. 4C, D and F) cells. However, simultaneous treatment with the antioxidant NAC as well as lupeol and beta-sitosterol significantly up-regulated the $H_2O_2$ induced downregulation of CDH1 gene both at m-RNA and E- cadherin protein level.

### *3.7. Increased Snail and Slug expression induced by $H_2O_2$ is downregulated by lupeol and beta sitosterol*

Snail and Slug are two important transcription factors which regulate the expression of CDH1 gene and facilitate the EMT [32]. To correlate this, expression of Snail and Slug was evaluated after $H_2O_2$ treatment. It was observed that MCF-7 and MDA-MB-231 cells after treatment with $H_2O_2$ shows significant higher expression of Snail and Slug as quantified by qRT-PCR against control (untreated). To confirm whether ROS acts as the major regulating factor in upregulation of Snail and Slug, cells were subsequently treated with NAC. Surprisingly, it was observed that the expression of Snail and Slug in $H_2O_2$ treated MCF-7 (Fig. 5A and B) and MDA-MB-231 (Fig. 5C and D) cells decreased significantly. Similar result was observed in lupeol and beta sitosterol treated cells. Thus, we assumed that ROS induced downregulation of CDH1 gene is regulated by Snail and Slug; whereas Lupeol and beta-sitosterol inhibits cellular migration through up-regulation of E-cadherin via downregulation of Snail and Slug.

### *3.8. Drugs inhibit the $H_2O_2$ induced downregulation of CDH1 by inhibiting the ERK pathway*

ERK pathway; being a crucial downstream signaling pathway in cell proliferation and cellular migration is reported to regulate expression of E-cadherin. Therefore, expression of E-cadherin along with Snail and Slug was evaluated in MCF-7 and MDA-MB-231 cells after treatment with ERK inhibitor U0126 at its sub-lethal dosage (Fig. 6A). Cellular migration was inhibited by U0126 (10 μM) in both MCF-7 (Fig. 6B) and MDA-MB-231 cells (Fig. 6C) through upregulation of E-cadherin and downregulation of Snail and Slug in MCF-7 (Fig. 6F) and MDA-MB-231 cells (Fig. 6H), suggesting ERK pathway is involved in Snail and Slug

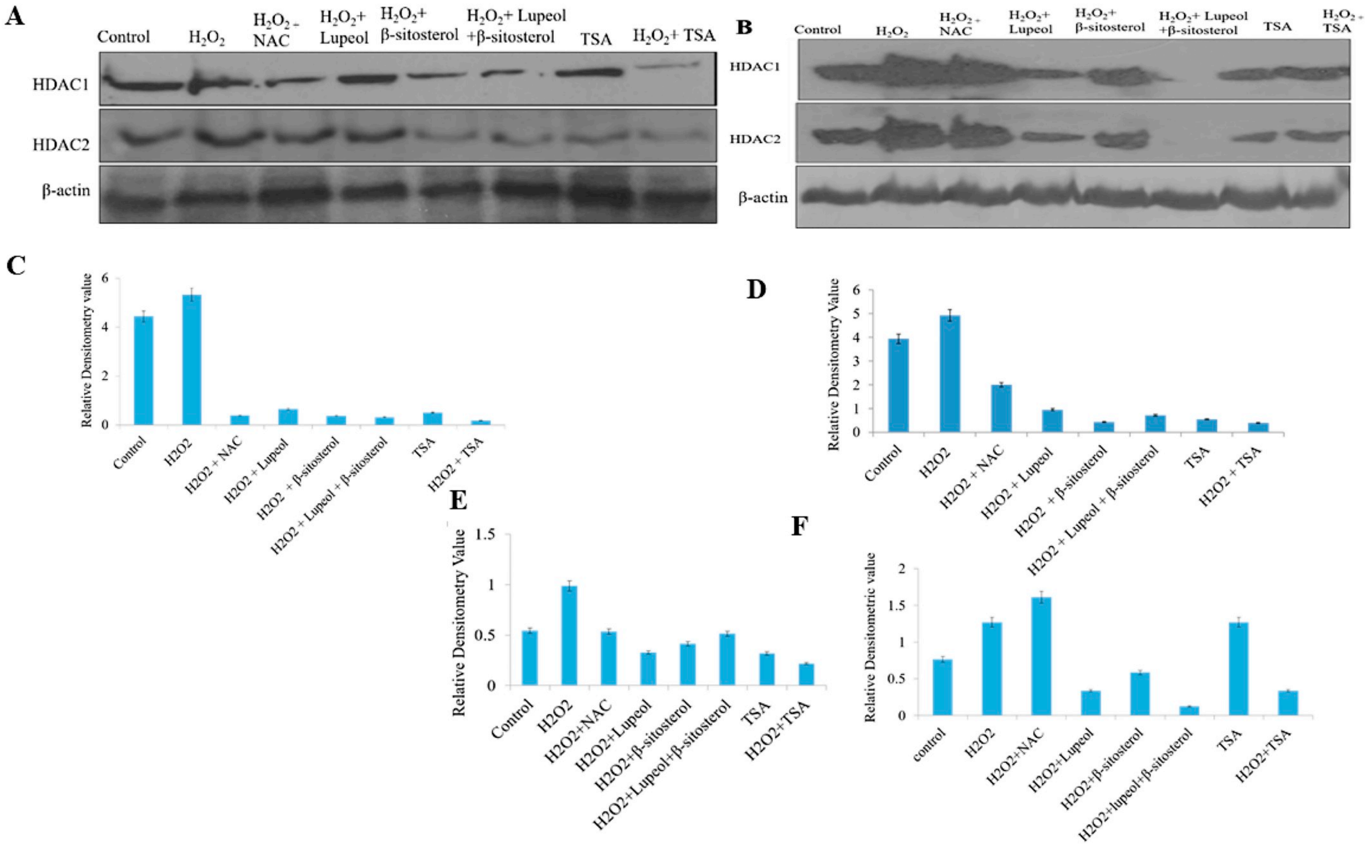

**Fig. 11.** Effect of $H_2O_2$ on histone modifications of CDH1 promoter region. Protein expression of HDAC1 and HDAC2 was analyzed by western blot analysis in $H_2O_2$ and $H_2O_2$ + drug treated MCF-7 cell lines (A); and in MDA-MB-231 cell lines (B). Beta-actin was used as internal control. Densitometric scanning of HDAC1(C) and HDAC2 (D) after normalization with beta-actin in MCF-7 and HDAC1(E) and HDAC2 (F) in MDA-MB-231 cell lines respectively. Enhanced expression of HDAC1 and HDAC2 was observed in both $H_2O_2$ treated MCF-7 and MDA-MB-231 cell lines as compared to control (untreated) one. Data are expressed as mean ± SEM., n = 3, $p < .05$.

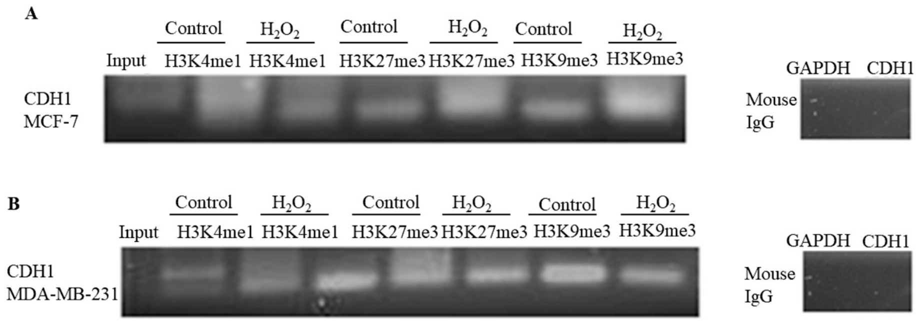

**Fig. 12.** Histone posttranslational modifications in promoter region of CDH1 gene was analyzed by chromatin immunoprecipitation assay (ChIP) using antibody against H3K4me1, H3K9me3 and H3K27me3 in MCF-7 cell line (A); and in MDA-MB-231 cell lines (B). Mouse IgG precipitated DNA used as negative control. ChIP analysis showed enrichment of H3K9me3 and H3K27me3 on promoter region of CDH1 gene supporting the gene silencing.

mediated down regulation of E-cadherin. In addition, $H_2O_2$ induced expression of Snail and Slug along with downregulation of E-cadherin were observed to be reversed by treatment with U0126 in MCF-7 (Fig. 6G) and MDA-MB-231 cells (Fig. 6I). This observation, further confirmed that down regulation of CDH1 by $H_2O_2$ induced upregulation of Snail and Slug is mediated through ERK pathway. Again to validate the data, amount of ROS production was measured in $H_2O_2$, $H_2O_2$ + U0126 and U0126 treated MCF-7 and MDA-MB-231 cells by fluorescence measurement using DCFDA dye. Important to note that there is no change in the reduction of free radical generation by same amount of $H_2O_2$ when U0126 were simultaneously used in both MCF-7 and MDA-MB-231 breast cancer cells. But sole treatment of U0126 reduced the amount of free radical generation in comparison to $H_2O_2$ and $H_2O_2$ + U0126 treated cells (Fig. 6E).

### *3.9. Expression profiling of DNA methyltransferases after $H_2O_2$ treatment*

Expression of DNMTs was analyzed both at mRNA and protein level to elucidate if ROS induced downregulation of CDH1 is correlated with DNMT activity. $H_2O_2$ treatment increases the expression of DNMT1 in MCF-7 (Fig. 7A, G and I) and MDA-MB-231(Fig. 7D, H and J) cells. Treatment of cells with $H_2O_2$ followed by NAC, lupeol and beta-sitosterol inhibited the DNMT1 expression in both mRNA and protein level. However, $H_2O_2$ has no such effect on DNMT3A and DNMT3B in both the MCF-7 (Fig. 7B, C) and MDA-MB-231 breast cancer cells (Fig. 7E, F).

### *3.10. Knockdown of DNMT1 enhances the expression of CDH1 gene*

It is evident from in silico analyses that higher expression of DNMT1 is associated with lower expression of E-cadherin in breast cancer. To validate this, we performed knockdown of DNMT1 by siDNMT1

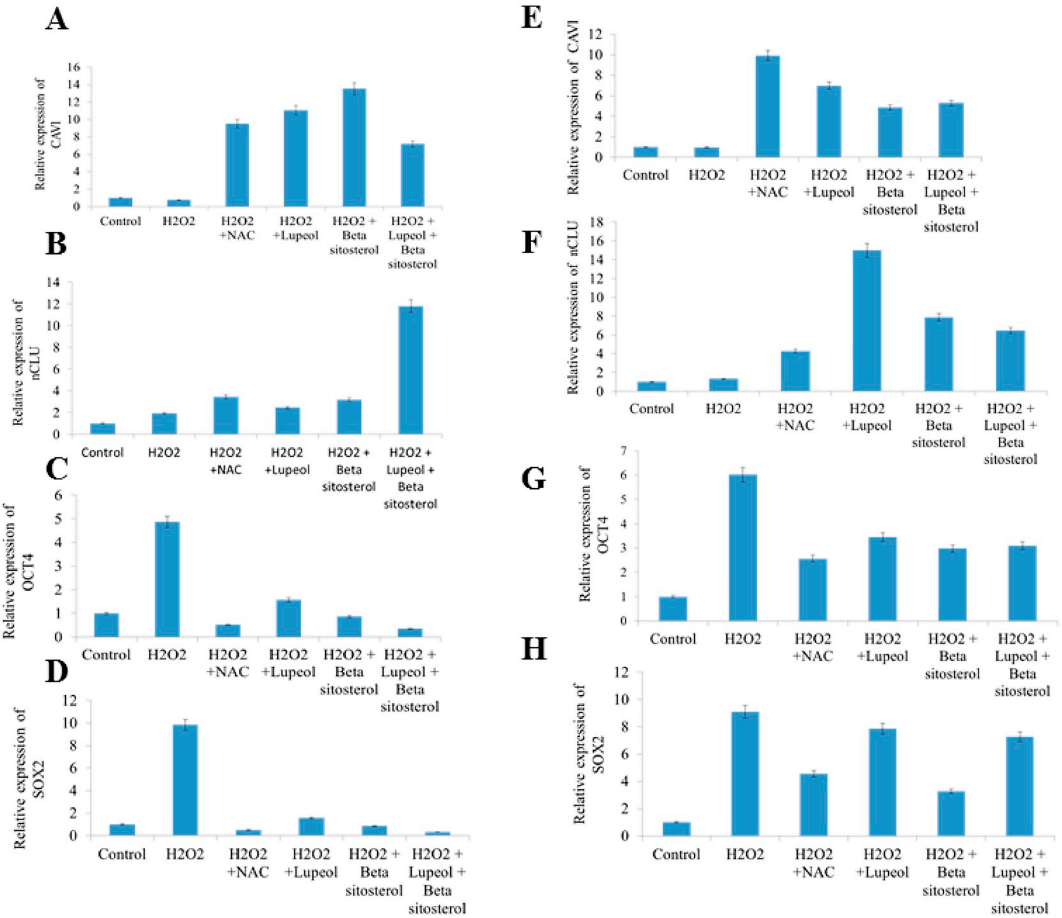

**Fig. 13.** Effect of $H_2O_2$ induced ROS on the expression profile of CAV1, nCLU, OCT4 and SOX2. qRT-PCR of CAV1(A), nCLU(B), OCT4(C), SOX2(D) in MCF-7 cell lines; and CAV1(E), nCLU(F), OCT4(G) and SOX2(H) in MDA-MB-231 cell lines. These genes are well documented for their regulation by modulations of the epigenetic marks. Data are expressed as mean ± SEM., n = 3, $p < .05$.

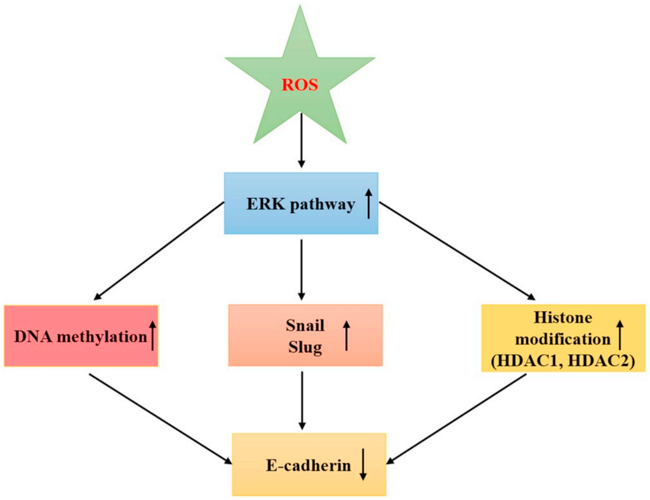

**Fig. 14.** Graphical Abstract (Epigenetic regulation of ROS induced down regulation of E-cadherin in breast cancer). ROS inactivates the CDH1 gene by Snail and Slug occupancy and activation of ERK pathway downstream components augmenting DNA methylation and Histone modification.

transfection in vitro cell culture and checked the expression status of E-cadherin. Upregulation of the E-cadherin expression both at m-RNA and protein level in MCF-7 (Fig. 8A, B and E) and MDA-MB-231 (Fig. 8C, D and F) cells were obtained by knockdown of DNMT1, suggesting the key role of DNMT1 in silencing of CDH1 gene and downregulation of E-cadherin protein.

### *3.11. Inhibition of DNA methylation reduce repressive effect of $H_2O_2$ upon CDH1*

To elucidate whether $H_2O_2$ induced downregulation of CDH1 is because of promoter methylation, MCF-7 and MDA-MB-231 cells were pretreated with DNMT inhibitor AZA for 72 h before $H_2O_2$ treatment. The expression of E-cadherin in protein level shows, AZA prevented the $H_2O_2$ induced downregulation of E-cadherin in MCF-7 (Fig. 9A, C) and MDA-MB-231 (Fig. 9B, D) cells. These results indicate that $H_2O_2$ induced downregulation of E-cadherin might be caused by promoter methylation. To confirm this, MS-PCR was done with methylation specific primer of E-cadherin promoter region. Significantly higher methylation of E-cadherin promoter in $H_2O_2$ treated cells compared to untreated was observed which was inhibited by DNMT inhibitor (AZA) as well as by NAC, lupeol and beta sitosterol in MCF-7 (Fig. 9E, G, I and K) and MDA-MB-231 cells (Fig. 9F, H, J and L). Thus, ROS induced downregulation of E-cadherin is regulated by promoter methylation.

### *3.12. ERK inhibitor decreases the expression of DNMTs at both transcript and protein level*

To check whether ROS induced down regulation of E-cadherin is directly through Snail and Slug mediated ERK pathway or by inducing DNMTs, the effect of ERK inhibitor U0126 on DNMTs was evaluated. It was found that, U0126 reduced the expression of DNMTs both at m-RNA and protein level in MCF-7 (Fig. 10A, C and E) and MDA-MB-231

(Fig. 10B, D and F) cells, indicating the expression of E-cadherin is regulated by DNMT1 through ERK pathway.

### 3.13. Role of HDACs in ROS induced downregulation of CDH1

To elucidate weather $H_2O_2$ induced down regulation of CDH1 is due to the enrichment of DNMT1 mediated DNA methylation or histone modification enzymes took part; expression of HDAC1 and HDAC2 was visualized after $H_2O_2$ treatment. DNMT1 interacts with HDAC1 to initiate gene silencing to repress transcription of genes [3,4,14]. It is quite convincing that $H_2O_2$ treatment enhanced the expression of both HDAC1 and HDAC2 in MCF-7 (Fig. 11A, C and D) and MDA-MB-231 (Fig. 11B, E and F) cells. Thus, we confirmed that not only DNMT1, but also HDAC1 may be involve in ROS induced downregulation of CDH1 in MCF-7 and MDA-MB-231 breast cancer cell lines.

### 3.14. DNA methylation of CDH1 gene promoter is associated with enrichment of H3K9me3 and H3K27me3

We reported earlier that miR-152 targets CDH1 mRNA and prevents production of E-cadherin protein. DNA methylation can dictate the repression of miR-152 gene even when there is coexistence of expressive histone modification H3K4me3 signals in the miR-152 gene promoter [26]. Hence, to check the fact whether $H_2O_2$ induced DNMT1 overexpression and DNA methylation of the promoter of CDH1 gene is associated with repressive marks of histone methylation, chromatin immunoprecipitation assay (ChIP) was performed. There was predominant K3K9me3 and H3K27me3 occupancy in CDH1 promoter in both MCF-7 (Fig. 12A) and MDA-MB-231 cells (Fig. 12B) with trace of H3K4me1.

### 3.15. Effect of hydrogen peroxide treatment on other genes

We tested the expression profile of a panel of genes those are regulated by epigenetic mechanisms, confirmed from our and other laboratories, before and after treatment with $H_2O_2$. The genes are; caveolin 1 (CAV1) involve in cell signaling; clusterin (CLU) involve in cellular physiology and function, and stem cell pluripotency inducers OCT4 and SOX2. Expression of CAV1 and nuclear CLU (nCLU) was decreased and OCT4 and SOX2 enhanced in both MCF-7 (Fig. 13 A, B, C and D) and MDA-MB-231 (Fig. 13 E, F, G and H) cells after $H_2O_2$ treatment. The decrease of CAV1 and nCLU reasonably supports compensation for signaling [7] and evading of apoptosis [6] respectively. Increased expression of OCT4 and SOX2 augurs the tumor-initiating capacities of the $H_2O_2$ treated cells where Snail/Slug favors EMT.

## 4. Discussion

There is large volume of data to understand the cause of cancer; however, insufficient! There are thousands of proteins as members of signaling pathways, cell metabolism and enzyme activity, as members of cytoskeletal assembly and membrane signaling and cell-cell attachment functions. We are working with a few of those proteins and regulatory mechanisms of their respective genes involved in dreadful diseases like cancer, including breast cancer. The roles of such proteins in morphological changes and function of cells of specific tissues and the roles of intrinsic metabolites are studied discreetly. Here we emphasized in testing the concerted role of loss of E-cadherin function in EMT and cell migration and the precise role of ROS in this scenario in activation of epigenetic modifiers like DNMT1 and HDAC1 and the downstream ERK component Snail and Slug.

Various aberrant epigenetic modulations like chromatin modifications, hypermethylation of CpG-island and transcriptional repressions are involved in silencing or inhibition of the expression of genes, including CDH1, CAV1, CLU in different cancers [6,7,33,34]. The impact of E-cadherin expression on overall survival of patients was analyzed using online KM plotter, suggesting poor prognosis owing to lower expression of E-cadherin in both estrogen positive and estrogen negative breast cancer patients (Fig. 1). To decipher the correlation of gene function and intrinsic metabolites we standardized first Cell survival and migration capability of breast cancer cell lines by ectopic application of ROS producer, like $H_2O_2$ and ROS scavengers like, NAC, Lupeol and beta-sitosterol (Figs. 2 and 3). Hydrogen peroxide ($H_2O_2$) is involved in various signal transduction pathways and cell fate decisions. The most prominent member of this class is the superoxide anion, largely produced by either the mitochondrial electron transport chain, in particular its complexes I, II and III, or by NAD(P)H oxidases. Here, we found $H_2O_2$ induced cellular migration via downregulation of CDH1 gene and absence of E-cadherin protein and enhanced expression of panel cooperatively active genes, including DNMT1, HDAC, Snail and Slug. Treatment of cells with $H_2O_2$ augmented vigorous ROS production which favors cellular proliferation and migration. The effects of $H_2O_2$ were reversed by treatment of cells with NAC, lupeol and beta-sitosterol, through scavenging ROS generated by $H_2O_2$ (Figs. 2 and 3). Question may arise that this regulation mechanism would not be the effect of ROS. Hence, we detected $H_2O_2$ induced ROS production by fluorescence measurement using DCFDA dye and clarified. Important to watch the reduction of free radical generation by the application of same amount of $H_2O_2$when antioxidants NAC, lupeol and beta sitosterol were simultaneously used in both MCF-7 and MDA-MB-231 breast cancer cells. MCF-7 and MDA-MB-231 cells were treated with $H_2O_2$ in the presence or absence of different drugs and NAC for 24 h and ROS level was detected using DCFDA dye by fluorescence microscopy as seen in the representative photographic images.

Deregulation of CDH1 gene is associated with cancer cell migration, loss of epithelial like properties with acquisition of mesenchymal properties through EMT and plays a vital role in tumor development and cancer metastasis [35]. In pathophysiological set up overproduction of ROS is associated with ERK activation during oncogenic transformation and ERK pathway is the most active signaling pathway involved in tumor metastasis [36–38]. We demonstrated that ectopic application of ROS reduced CDH1 mRNA and E-cadherin protein (Fig. 4) and overexpression of Snail and Slug (Fig. 5). Snail and Slug are downstream effector of ERK pathway and our experiment with U0126 treatment of cells demonstrated E-cadherin expression inhibition of cell migration (Fig. 6).

The cooperative effect of reversible DNA methylation and histone modifications plays significant role in regulating gene silencing/expression during tumor development and cancer progression [4,16,39–41]. Histone deacetylation allows DNA to tightly wrap around the histone core and hence prevents binding of transcription factor on promoter site to inhibit transcription [16,42–45]. Consistent with these findings our results show that $H_2O_2$ potentiates down regulation of CDH1 by DNA methylation through over expression of DNMT1 (Fig. 7). This was further confirmed by DNMT1 knockdown upon siDNMT1 treatment (Fig. 8) and experiments with DNMT inhibitor AZA treatment (Fig. 9) and up-regulation of CDH1 at mRNA and E-cadherin protein level in both MCF-7 and MDA-MB-231 cell lines. Additionally, from the MS-PCR data it is apparent that $H_2O_2$ induced DNMT1 mediated promoter methylation of CDH1 was abolished by application of DNMT inhibitor AZA, and free radical scavengers NAC, lupeol and beta-sitosterol (Fig. 9). These observations elucidate, ROS induced repression of CDH1 is regulated by covalent DNA methylation. We and others reported earlier that DNMT1 plays a major role in modulation of expression of E-cadherin [24,46,47]. However, whether there is any correlation between ERK pathway and DNA methylation in repression of CDH1?

To address this, we demonstrated and experiment with U0126 treatment of cells which reduced expression of DNMT1, Snail and Slug with the enhanced expression of E-cadherin (compare Figs. 6 and 10). DNA methylation and histone deacetylation reported to regulate gene expression in a cooperative manner and our data is in full agreement with previous observations. Besides, Trichostatin A (TSA) was also used as a reference HDAC inhibitor. Thus, $H_2O_2$ induced down regulation of E-cadherin is also significantly cooperatively regulated by DNA methylation and histone deacetylation (Fig. 11). In addition to DNA methylation, the role of histone methylation in regulation of $H_2O_2$ induced downregulation of E-cadherin was evaluated. Enrichment of H3K9me3 and H3K27me3 repressive modifications with trace of H3K4me1 only on promoter site of CDH1, which suggests the significant role of histone 3 methylation in down regulation of CDH1 (Fig. 12) (for further discussion see, [48–50]). Besides, $H_2O_2$ induced ROS also regulates the expression of CAV1, nCLU, OCT4 and SOX2 in both MCF-7 and MDA-MB-231 cells (Fig. 13).

Taken together the result obtained here suggests that, E-cadherin possesses a key role in development and progression of breast cancer. The expression of E-cadherin is epigenetically regulated during initiation and progression of breast cancer. The role of ROS in regulation of CDH1 gene is basically modulated by both DNA methylation and histone methylation and deacetylation modifications epigenetic pathways and activation of ERK/Snail/Slug axis (Fig. 14). In conclusion, our work; (i) documented that free radical scavengers would be effective for therapeutic intervention of breast cancer, (ii) this is first ever report that cells exploit cooperative actions of ROS and ERK signaling to facilitate epigenetic gene repression and strengthen the fact that elevated moderate levels of ROS act as key cell signaling molecules, and (iii) widens the amplitude of cancer epigenome and scope of investigation on epigenetics and cancer.

## Conflicts of interest

The authors have no conflicts of interest.

## Transparency document

The Transparency document associated with this article can be found, in online version.

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